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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Offi

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Novel cxcl8 antagonists

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NOVEL CXCL8 ANTAGONISTS

FIELD OF THE INVENTION

The invention relates to structure and the properties of novel antagonists of the chemokine CXCL8.

BACKGROUND OF THE INVENTION

Chemokines are secreted pro-inflammatory proteins of small dimensions (70-130 amino acids) mostly involved in the directional migration and activation of cells, especially the extravasation of leukocytes from the blood to tissue localizations needing the recruitment of these cells (Baggiolini M et al., 1997; Fernandez EJ and Lolis E, 2002).

Depending on the number and the position of the conserved cysteines in the sequence, chemokines are classified into C, CC, CXC and CX_3C chemokines. A series of membrane receptors, all heptahelical G-protein coupled receptors, are the binding partners that allow chemokines to exert their biological activity on the target cells, which present specific combinations of receptors according to their state and/or type. The physiological effects of chemokines result from a complex and integrated system of concurrent interactions: the receptors often have overlapping ligand specificity, so that a single receptor can bind different chemokines, as well a single chemokine can bind different receptors.

Usually chemokines are produced at the site of an injury, inflammation, or other tissue alteration in a paracrine or autocrine fashion. However, cell-type specific migration and activation in inflammatory and immune processes is not the sole activity

of chemokines, but other physiological activities, such as hematopoiesis or angiogenesis, appears to be regulated by certain of these proteins.

Even though there are potential drawbacks in using chemokines as therapeutic agents (tendency to aggregate and promiscuous binding, in particular), chemokines offer the possibility for therapeutic intervention in pathological conditions associated to such processes, in particular by inhibiting specific chemokines and their receptors at the scope to preventing the excessive recruitment and activation of cells, in particular leukocytes (Proudfoot A, 2000; Baggiolini M, 2001; Haskell CA et al., 2002; Godessart N and Kunkel SL, 2001).

Studies on structure-activity relationships indicate that chemokines have two main sites of interaction with their receptors, the flexible amino-terminal region and the conformationally rigid loop that follows the second cysteine. Chemokines are thought to dock onto receptors by means of the loop region, and this contact is believed to facilitate the binding of the amino-terminal region that results in receptor activation. This importance of the amino-terminal region has been also demonstrated by testing natural and synthetic chemokines in which this domain is modified or shortened. This processing, following proteolytic digestion, mutagenesis, or chemical modification of amino acids, can either activate or render these molecules completely inactive, generating compounds with agonistic and/or antagonistic activity. Thus, chemokines with specific modifications in the amino-terminal region are considered having therapeutic potential for inflammatory and autoimmune diseases (Schwarz and Wells, 1999).

molecules are formed by disaccharide repeats (such as heparin, chondroitin sulfate, heparan sulfate, dermatan sulfate, and hyaluronic acid) and naturally occur on cell surfaces, in the extracellular matrix, or in the circulation. They can be present in isolated forms or linked to proteins (Proteoglycans, or PGs) following the posttranslational addition of GAGs at serine residues.

Chemokines, as the other GAG-binding proteins, have basic residues (mainly Arginine and Lysine) clustered in short portions of their sequence which are suitable for this purpose but such motifs are structured in different manner for each c hemokine, or group of highly homologous chemokines. Some of these GAG-binding sites have been associated to specific consensus, such as BBXB motifs (where B represents a basic residue, and X any other residue) or other arrangements (Kuschert G et al., 1999; Proudfoot A et al., 2001; Proudfoot A et al., 2003).

The main consequence of this interaction is the aggregation of the chemokines, a state which is believed to provide a protection from proteolysis, as well as a mechanism for the controlled and gradient-generating release of the chemokines, participating to the recognition and to the presentation of chemokines to the receptors as oligomers (Hoogewerf AJ et al., 1997; Kuschert G et al., 1999). The interaction with GAGs and the formation of these gradients has been clearly demonstrated for many chemokines, and the relative affinity has been measured. Therefore, it has been suggested that also the modulation of such interactions may represent a therapeutic approach in inflammatory disease (Ali S et al., 2001; Patel D et al., 2001).

Means to achieve a therapeutic effect on the basis of the GAGs-chemokines interactions known in the art involve the generation of GAGs analogs modulating the interaction between endogenous GAGs and chemokines (WO 94/20512), the use of heparanase for eliminating GAGs (WO 97/11684), the administration of chemokine-

GAGs complexes (WO 99/62535), the modification of GAGs binding domain with polymers (WO 02/04015), or the substitution of residues involved in GAG-binding activity (WO 02/28419, WO 03/051921).

Even though extensive studies have been performed on some chemokines, it is well established that is not possible to anticipate, on the basis of the sequence homology with chemokine having limited similarity or known GAGs-binding protein motifs, which specific basic residues have to be modified with non-conservative substitutions to impair GAG-binding, since there is a significant structural diversity of GAG-binding domains amongst the chemokine protein family (Lortat-Jacob H et al., 2002).

Amongst chemokines, CXCL8 (also known as Interleukin-8, IL-8, monocyte-derived neutrophil chemotactic factor, MDNCF, Neutrophil-Activating Protein 1, NAP-1, lymphocyte-derived neutrophil-activating factor, LYNAP, neutrophil-activating factor, NAF, granulocyte chemotactic protein 1, GCP-1, Emoctakin) is known as a potent chemotactic inflammation-mediating factor exerting its activity not only on neutrophils, but also on lymphocytes, monocytes, endothelial cells, and fibroblasts (Mukaida N, 2003; Shi Q et al., 2001; Zeilhofer HU and Schorr W, 2000; Atta-ur-Rahman H and Siddiqui RA, 1999).

CXCL8 is produced from various types of cells in response to a wide variety of inflammatory stimuli: cytokines, microbial products, environmental changes (such as hypoxia, acidosis, hyperglycemia, hyperosmotic pressure, high cell density, hyperthermia, radiation, chemotherapeutic agents, or reperfusion). It has also been

In connection to its receptors CXCR2 and CXCR1, CXCL8 mediates several intracellular events associated to numerous physiologic and pathophysiologic processes, such as in host defense mechanism. CXCL8 activates signal trans duction processes leading to desensitization, internalization, and recycling of CXCR2/CXCR1.

The discovery of these biological functions suggests that CXCL8 is an important mediator of various pathological conditions such as chronic inflammation and cancer, implying that blockade of its actions could be exploited for therapeutic purposes. The literature provides many examples of molecules inhibiting CXCL8, including CXCL8-derived mutants or peptides (WO 91/08231, WO 93/11159, WO 96/09062; Moser B et al., 1993).

CXCL8-GAGs interactions have been studied, also by the means of CXCL8 mutants which are truncated or have single amino acid substitutions, to characterize their involvement in CXCL8 properties, such as the retention in specific tissues (Frevert C et al., 2003; Frevert C et al., 2002) and receptor / heparin binding (Goger B et al., 2002; Spillmann D et al., 1998; Kuschert GS, et al., 1998; Kuschert GS, et al., 1997; Hoogewerf AJ et al., 1997; Skelton N et al., 1999; Dias-Baruffi M et al., 1998; Witt D and Lander A, 1994; Webb L et al., 1993), identifying GAG-binding motifs in the 20's loop and C-terminal regions of CXCL8. However, none of these approaches identified CXCL8 variants having the *in vivo* antagonistic effects against CXCL8.

SUMMARY OF THE INVENTION

It has been found that specific combinations of basic residues in the carboxyl-terminus of human CXCL8 can be substituted to generate CXCL8 antagonists.

The elimination of these basic residues by non-conservative substitutions (for example, with Alanines) leads to the generation of CXCL8 mutants having antagonistic

activities against CXCL8 *in vivo*. Compounds prepared in accordance with the present invention can be used to block the activity of CXCL8 on CXCL8-binding cells, thereby providing therapeutic compositions for use in the treatment of CXCL8-related diseases.

Other features and advantages of the invention will be apparent from the following detailed description.

DESCRIPTION OF THE FIGURES

Figure 1: amino acid sequences of mature human CXCL8 (hCXCL8-WT; SEQ ID NO: 1), and of the mutants generated on the basis of these sequence, CXCL8-1B3 (SEQ ID NO: 2) and CXCL8-2B3 (SEQ ID NO: 3), which have been expressed and tested as described in the Examples (mutated amino acids are underlined; the numbering is based on the mature human sequence). The cluster of basic amino acids in CXCL8 sequence is boxed.

Figure 2: A) Graph summarizing the effects of CXCL8 and of CXCL8 mutants in the *in vivo* peritoneal cell recruitment model, compared with baseline, at 4 hours (data expressed as mean total cells ± standard error; n=3 mice per group). B) Graph summarizing the inhibiting effects of CXCL8 mutants on CXCL8-induced cell recruitment in the peritoneal cavity, compared to saline treatment (data expressed as mean total cells ± standard error; n = 3 mice per group. $p < 0.05$ *, $p < 0.001$ ***).

DETAILED DESCRIPTION OF THE INVENTION

Glycine, Serine, Threonine, Proline, Glutammic Acic, Glutamine, Aspartic Acid, or Asparagine. A third basic residues that can be additionally mutated in the same way in preferred mutants, can be chosen amongst the more proximal (Arginine 60 and Lysine Arginine 68).

More in particular, the present patent application provides data on the antagonistic activities of novel recombinant CXCL8 mutants, having specific combinations of basic residues substituted with Alanines, against CXCL8. These mutants have the sequence of CXCL8-1B3 (SEQ ID NO: 2), or of CXCL8-2B3 (SEQ ID NO: 3), wherein, respectively, Arginine60-Lysine64-Lysine67 and Lysine64-Lysine67-Arginine68 are mutated to Alanine.

CXCL8 mutants prepared in accordance with the present invention can be used to block the activity of CXCL8*in vivo*, thereby providing therapeutic compositions for use in the treatment of CXCL8-related diseases, due to excessive or uncontrolled CXCL8 production.

The amino acid replacing the specific combinations of basic residue is preferably a non-polar, small amino acid like Alanine or Glycine, but other amino acids are appropriate, provided that they have a charge and dimension which are incompatible with GAG-binding and, at the same time, poorly interfere with other properties of the protein. Amino acids suitable for the substitutions are Serine, Threonine, Proline, Glutammic Acic, Glutamine, Aspartic acid, or Asparagine.

Further objects of the present invention are antagonists of CXCL8 antagonsts selected from:

a) active mutants of the above defined antagonists in which one or more amino acid residues have been added, deleted, or substituted;

b) active peptide mimetics designed on the sequence and/or the structure of polypeptides or peptides of (a);

c) active polypeptides or peptides comprising the amino acid sequence of (a) or (b), and an amino acid sequence belonging to a protein sequence other than human mature CXCL8;

d) active fractions, precursors, salts, derivatives, conjugates, or complexes of (a), (b), or (c).

The term "active" means that such alternative compounds should maintain, or even potentiate, the antagonistic properties of the CXCL8 mutants of the present invention, and should be as well pharmaceutically acceptable and useful.

Amongst the active mutants of the (a) category, natural or artificial analogs of said sequence, wherein one or more amino acid residues have been added, deleted, or substituted, can be included, provided they display the same biological activity characterized in the present invention at comparable or higher levels, as determined by means known in the art and disclosed in the Examples below.

Natural analogs are intended the corresponding sequences of CXCL8 in other organisms, like mouse. Artificial analogs are intended peptides and polypeptides generated by site-directed mutagenesis techniques, combinatorial technologies at the level of encoding DNA sequence (such as DNA shuffling, phage display/selection), or by computer-aided design studies, or any other known technique suitable thereof, which provide a finite set of substantially corresponding mutated or shortened peptides or polypeptides. These alternative molecules can be routinely obtained and tested by

For example, specific artificial mutants may have one or more amino acids being added, deleted, or substituted in the amino-terminal region known to affect receptor binding. In particular, these mutations may involve one or more of the first six amino acids of the mature human CXCL8 positioned in the amino-terminal region, just before the conserved CXC motif (fig. 1).

In accordance with the present invention, preferred changes in these active mutants are commonly known as "conservative" or "safe" substitutions, and involve non-basic residues. Conservative amino acid substitutions are those with amino acids having sufficiently similar chemical properties, in order to preserve the structure and the biological function of the molecule. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their functio n, particularly if the insertions or deletions only involve a few amino acids, e.g., under ten, and preferably under three, and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used to detect functional and structural CXCL8 homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups for the substitutions are those defined in Table I.

Specific variants of the CXCL8 antagonists of the invention can be obtained in the form of peptide mimetics (also called peptidomimetics) of the disclosed mutants of CXCR3 binding chemokines, in which the nature of peptide or polypeptide has been chemically modified at the level of amino acid side chains, of amino acid chirality, and/or of the peptide backbone. These alterations are intended to provide antagonists with improved preparation, potency and/or pharmacokinetics features.

For example, when the peptide is susceptible to cleavage by peptidases following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a non-cleavable peptide mimetic can provide a peptide more stable and thus more useful as a therapeutic. Similarly, the replacement of an L-amino acid residue is a standard way of rendering the peptide less sensitive to proteolysis, and finally more similar to organic compounds other than peptides. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4-dinitrophenyl. Many other modifications providing increased potency, prolonged activity, easiness of purification, and/or increased half-life are known in the art (WO 02/10195; Villain M et al., 2001).

Preferred alternative, "synonymous" groups for amino acids derivatives included in peptide mimetics are those defined in Table II. A non-exhaustive list of amino acid derivatives also include aminoisobutyric acid (Aib), hydroxyproline (Hyp), 1,2,3,4-tetrahydro-isoquinoline-3-COOH, indoline-2carboxylic acid, 4-difluoro-proline, L-thiazolidine-4-carboxylic acid, L-homoproline, 3,4-dehydro-proline, 3,4-dihydroxy-

particular, the amino acid derivative may contain substituted or non-substituted alkyl moieties that can be linear, branched, or cyclic, and may include one or more heteroatoms. The amino acid derivatives can be made de novo or obtained from commercial sources (Calbiochem-Novabiochem AG, Switzerland; Bachem, USA).

Various methodologies for incorporating unnatural amino acids derivatives into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are disclosed in the literature (Dougherty DA, 2000). Techniques for the synthesis and the development of peptide mimetics, as well as non-peptide mimetics, are also well known in the art (Hruby VJ and Balse PM, 2000; Golebiowski A et al., 2001).

Still specific variants of the CXCL8 antagonists of the invention are the ones comprising one of the amino acid sequence as defined above and an amino acid sequence belonging to a protein sequence other than the human mature CXCL8. This heterologous latter sequence should provide additional properties without impairing significatively the antagonistic activity, or improving GAGs-binding properties. Examples of such additional properties are an easier purification procedure, a longer lasting half-life in body fluids, an additional binding moiety, the maturation by means of an endoproteolytic digestion, or extracellular localization. This latter feature is of particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows the molecules defined as CXCL8 antagonists in this invention to be localized in the space where not only where the isolation and purification of these polypeptides is facilitated, but also where CXCL8 and its receptors naturally interact.

Design of the moieties, ligands, and linkers, as well methods and strategies for the construction, purification, detection and use of fusion proteins are widely discussed

in the literature (Nilsson J et al., 1997; "Applications of chimeric genes and hybrid proteins" Methods Enzymol. Vol. 326-328, Academic Press, 2000; WO 01/77137). Additional protein sequences which can be used to generate the antagonists of the present invention are chosen amongst extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins. The choice of one or more of these sequences to be fused to the CXCL8 mutant of th einvention is functional to specific use and/or purification protocol of said agent.

The polypeptides and the peptides of the present invention can be in other alternative forms which can be preferred according to the desired method of use and/or production, for example as active fractions, precursors, salts, derivatives, conjugates or complexes.

The term "fraction" refers to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the amino-/ or carboxy -terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

Useful conjugates or complexes of the antagonists of the present invention can be generated, using molecules and methods known in the art of the interaction with receptor or other proteins (radioactive or fluorescent labels, biotin), therapeutic efficacy (cytotoxic agents), or improving the agents in terms of drug delivery efficacy, such as polyethylene glycol and other natural or synthetic polymers (Pillai O and Panchagnula R, 2001). In the latter case, the antagonists may be produced following a site-directed modification of an appropriate residue, in an internal or terminal position.

Residues can be used for attachment, provided they have a sidechain amenable for polymer attachment (i.e., the side chain of an amino acid bearing a

functional group, e.g., lysine, aspartic acid, glutamic acid, cysteine, histidine, etc.). Alternatively, a residue at these sites can be replaced with a different amino acid having a side chain amenable for polymer attachment. Also, the side chains of the genetically encoded amino acids can be chemically modified for polymer attachment, or unnatural amino acids with appropriate side chain functional groups can be employed. Polymer attachment may be not only to the side chain of the amino acid naturally occurring in a specific position of the antagonist or to the side chain of a natural or unnatural amino acid that replaces the amino acid naturally occurring in a specific position of the antagonist, but also to a carbohydrate or other moiety that is attached to the side chain of the amino acid at the target position.

Polymers suitable for these purposes are biocompatible, namely, they are non-toxic to biological systems, and many such polymers are known. Such polymers may be hydrophobic or hydrophilic in nature, biodegradable, non-biodegradable, or a combination thereof. These polymers include natural polymers (such as collagen, gelatin, cellulose, hyaluronic acid), as well as synthetic polymers (such as polyesters, polyorthoesters, polyanhydrides). Examples of hydrophobic non-degradable polymers include polydimethyl siloxanes, polyurethanes, polytetrafluoroethylenes, polyethylenes, polyvinyl chlorides, and polymethyl methaerylates. Examples of hydrophilic non-degradable polymers include poly(2-hydroxyethyl methacrylate), polyvinyl alcohol, poly(N-vinyl pyrrolidone), polyalkylenes, polyacrylamide, and copolymers thereof. Preferred polymers comprise as a sequential repeat unit ethylene oxide, such as polyethylene glycol (PEG).

Such modification acts to provide the protein in a precursor (or "pro-drug") form, that, upon degradation of the linker releases the protein without polymer modfication.

The antagonists of the invention may be prepared by any procedure known in the art, including recombinant DNA-related technologies, and chemical synthesis technologies.

Another object of the invention are the DNA molecules comprising the DNA sequences coding for the antagonists of CXCR3-binding CXC chemokines described above, including nucleotide sequences substantially the same.

"Nucleotide sequences substantially the same" includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences.

Still another object of the invention are expression vectors which comprise the above DNAs, host cells transformed with such vectors, and the process of preparation of the antagonists described above, comprising culturing these transformed cells and collecting the expressed proteins. When the vector expresses the antagonists as a fusion protein with extracellular, export signal, or signal-peptide containing proteins, CXCL8 antagonists can be secreted in the extracellular space, and can be more easily collected and purified from cultured cells in view of further processing or, alternatively, the cells can be directly used or administered.

These other objects of the invention can be achieved by combining the disclosure provided herein with the knowledge of common molecular biology techniques. Many books and reviews provides teachings on how to clone and produce recombinant proteins using vectors and Prokaryotic or Eukaryotic host cells, such as some titles in the series "A Practical Approach" published by Oxford University Press ("DNA Cloning

2: Expression Systems", 1995; "DNA Cloning 4: Mammalian Systems", 1996; "Protein Expression", 1999; "Protein Purification Techniques", 2001).

The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable episomal or non-/homologously integrating vectors, which can be introduced in the appropriate host cells by any suitable means (transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.) to transform them. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector, may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

The vectors should allow the expression of the isolated or fusion protein including the antagonist of the invention in the prokaryotic or eukaryotic host cell under the control of transcriptional initiation / termination regulatory sequences, which are chosen to be constitutively active or inducible in said cell. A cell line substantially enriched in such cells can be then isolated to provide a stable cell line.

For eukaryotic hosts (e.g. yeasts, insect or mammalian cells), different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived form viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are

cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to an auxotropic host, biocide resistance, e.g. antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese Hamster Ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences in cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the carboxy-terminus of the peptide to be synthetized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the carboxy-terminus to the amino-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the

peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl2-Bzl (2,6-dichlorobenzyl) for the amino groups; NO2 (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups); and tBu (t-butyl) for the hydroxyl groups). After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Totally synthetic chemokines are disclosed in the literature (Dawson PE et al., 1994;Brown A et al., 1996).

Purification of the natural, synthetic or recombinant antagonists of the invention can be carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies or affinity groups, which bind the target protein and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be

used. The elution can be carried using a water-acetonitrile-based solvent commonly employed for protein purification.

Another object of the invention is represented by purified preparations of said CXCL8 antagonists. Purified preparations, as used herein, refers to the preparations which are at least 1% (by dry weight), and preferably at least 5%, of said antagonists.

Another object of the present invention is the use of CXCL8 antagonists as medicaments, in particular as the active ingredients in pharmaceutical compositions for the treatment or prevention CXCL8-related diseases.

Another object of the present invention are pharmaceutical composition containing, as active ingredient, an antagonist of CXCL8 in the forms defined above: proteins, peptide mimetics, derivatives, precursors, as well as DNA coding or cells expressing them. The processes for the preparation of such pharmaceutical compositions comprise combining the CXCL8 antagonist together with a pharmaceutically acceptable carrier.

The pharmaceutical compositions may contain, in combination with the CXCL8 antagonist of the invention, suitable pharmaceutically acceptable carriers, biologically compatible vehicles and additives which are suitable for administration to an animal (for example, physiological saline) and eventually comprising auxiliaries (like excipients, stabilizers, adjuvants, or diluents) which facilitate the processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration. For example, the use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. The effective amount will depend on the route of administration and the condition of the patient.

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution. Carriers can be selected also from starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the various oils, including those of petroleum, animal, vegetable or synthetic origin (peanut oil, soybean oil, mineral oil, sesame oil).

Any accepted mode of administration can be used and determined by those skilled in the art to establish the desired blood levels of the active ingredients. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. The pharmaceutical compositions of the present invention can also be administered in sustained or controlled release dosage forms, including depot injections, osmotic pumps, and the like, for the prolonged administration of the polypeptide at a predetermined rate, preferably in unit dosage forms suitable for single

solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99.99 percent, preferably from about 20 to 75 percent of active compound together with the excipient.

It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, freq uency of treatment, and the nature of the effect desired. The dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. The total dose required for each treatment may be administered by multiple doses or in a single dose. The pharmaceutical composition of the present invention may be administered alone or in conjunction with other therapeutics directed to the condition, or directed to other symptoms of the condition. Usually a daily dosage of active ingredient is comprised between 0.01 to 100 milligrams per kilogram of body weight per day. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

Another object of the present invention is also the method for treating or preventing CXCL8-related diseases comprising the administration of an effective amount of an antagonist of CXCL8 of the present invention.

The wording "CXCL8-related diseases" indicate any disease due to an excessive or uncontrolled CXCL8 production, leading to a massive neutrophil / T-cell infiltration or neovascular growth, and wherein the administration of a CXCL8 antagonist may provide a beneficial effect. A non-exhaustive list of such chronic, acute, or inherited diseases includes: hyperproliferative diseases, auto-/immune diseases, inflammatory diseases, bacterial/fungal//protozoal/viral infections, cardiac and vascular diseases. Specific examples of such diseases are: leiomyomas, adenomas, lipomas, hemangiomas, fibromas, vascular occlusion, restenosis, atherosclerosis, cancer, neoplasia, carcinoma, psoriasis, atopic dermatitis, rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, adult respiratory distress syndrome, inflammatory bowel disease, Crohn's disease, ulcerative colitis, stroke, septic shock, endotoxic shock, gram negative sepsis, toxic shock syndrome, cardiac and renal reperfusion injury, glomerulonephritis, thrombosis, graft vs. host reaction, A1zheimer's disease, allograft rejections, malaria, restinosis, angiogenesis, atherosclerosis, osteoporosis.

The therapeutic applications of the CXCL8 antagonists of the invention and of the related reagents can be evaluated (in terms or safety, pharmacokinetics and efficacy) by the means of the *in vivo* or *in vitro* assays making use of animal cell, tissues and models (Coleman R et al., 2001; Li A, 2001; Methods Mol. Biol vol. 138, "Chemokines Protocols", edited by Proudfoot A et al., Humana Press Inc., 2000; Methods Enzymol,

substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

EXAMPLES

Example 1: preparation and characterization of the CXCL8 mutants

Materials and methods

Expression of the human CXCL8 mutants.

Mature human CXCL8 and CXCL8 mutants were expressed in the yeast *Pichia pastoris* using the vector pPIC9K (Invitrogen) that allows the secretion of the cloned protein using the *S. cerevisiae* Mat α-factor pre-pro signal peptide.

The CXCL8 mutants were generated by "megaprimer" PCR mutagenesis (Sarkar G and Sommer S,1990) of the DNA sequence coding for human CXCL8 (IL-8; SWISSPROT Acc. N° P10145), and in particular for the mature form, corresponding to the segment 28-99 of the precursor molecule, containing 72 amino acids.

The mutations were confirmed by sequencing. The pPIC9K-based vectors for expressing recombinant human CXCL8 (CXCL8-WT; fig. 1; SEQ ID NO: 1), CXCL8-1B3 (CXCL8-1B3; fig. 1; SEQ ID NO: 2), and CXCL8-2B3 (CXCL8-2B3; fig. 1; SEQ ID NO: 3) were used to transform into *Pichia pastoris* (strain GS115-His$^-$) by electroporation. His$^+$ transformants were selected on minimal medium and screened for resistance to 1 mg/ml Geneticin (G418). G418-resistant clones were analyzed for secretion of the recombinant CXCL8 variants by small-scale induction with 0.5% methanol in shake flasks, and analysed by Coomassie Blue-stained SDS-PAGE.

The purification of the recombinant proteins was performed by removing the supernatant of the culture and adjusting the pH of the solution to 4.5 with acetic acid, and the conductivity to 20 mS by dilution with H_2O. The solution was applied to a HiLoad S 26/10 column previously equilibrated in 20 mM sodium acetate, (pH 4.5) and protein was eluted with a linear 0-2M NaCl gradient in the same buffer. The fractions containing the recombinant protein were pooled, dialysed against two changes of 1 % acetic acid, and finally against 0.1% trifluoroacetic acid, and then lyophilised. The authenticity of the protein was verified by mass spectrometry.

Peritoneal cellular recruitment

Female Balb/C mice (Janvier, France) of 8 to 12 weeks were housed under normal animal holding conditions with a standard 12-h light/dark cycle and free access to food and water.

Groups composed of 3 mice were injected intraperitoneally with 200 µl of saline (sterile LPS-free NaCl 0.9% (w/v)), or of this solution containing of CXCL8 or of one its mutants at 10 µg per injection.

For studies investigating the inhibitory effects of CXCL8 mutants on CXCL8-induced peritoneal cell chemotaxis, these molecules were administered intraperitoneally 30 minutes before the intraperitoneal injection of CXCL8. All the molecules were administered at the concentration and in buffer above indicated.

Peritoneal lavages to assess cell recruitment were performed at 4 hours after the chemokine, or chemokine mutant, final injection as follows. Mice were sacrificed by asphyxiation with rising concentrations of CO_2 in a plexiglass box. Skin was cleaned with

supernatant discarded and the resultant cell pellet was resuspended by gentle multiple pipetting in 1 ml PBS. 10 µl cell suspension was stained with 90 µl trypan blue and total cell counts were enumerated with a Neubauer haemocytometer by counting 4 areas each of 1 mm^2. The mean of the 4 counts was taken, multiplied by the dilution factor of 10, and multiplied again by 10 to give the number of cells per µl, according to the directions for use accompanying the haemocytometer. Finally the total value was multiplied by 1000 (to equal 1 ml) to arrive at the total cell number recovered.

Results

Human CXCL8 was expressed in two mutated forms to identify the sequence and the properties of non-heparin binding variants. Target of the mutations were basic residues clustered in the C-terminus of human mature CXCL8 (SEQ ID NO: 1), either Arginine60-Lysine64-Lysin 67 (CXCL8-1B3; SEQ ID NO: 2) or Lysine64-Lysine 67-Arginine 68 (CXCL8-2B3; SEQ ID NO: 3). The residues located in this region are involved in heparin binding but they are spatially distinct from those involved in receptor binding (Kuschert G et al., 1998)

The ability of the mutants to modulate *in vivo* chemotaxis was tested in the peritoneal cell recruitment model to examine whether two different manners for disrupting the GAG binding sites of CXCL8 may lead to molecules affecting cell recruitment *in vivo*.

Neither CXCL8 mutants induced a significant increase over baseline compared with the increase in cells recovered from mice treated with the parent chemokine (Fig. 2A), but both molecules show to block CXCL8-mediated recruitment of cells in the peritoneal cell chemotaxis model with a high degree of significance (Fig. 2B).

In conclusion, CXCL8-1B3 and CXCL8-2B3 shows no recruitment proprieties of its own, but considerable antagonistic activities over CXCL8-WT, with respect of

cellular recruitment induction into the peritoneum, when prior a dministered at the same dose (10 µg/mouse). Therefore, the combined substitution of specific basic residues in the C-terminal region of CXCL8 produces CXCL8 antagonist capable of inhibiting *in vivo* the cellular recruitment induced by CXCL8.

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TABLE I

AminoAcid	Synonymous Group	More Preferred Synonymous Groups
Ser	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Arg	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Leu	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Pro	Gly, Ala, Ser, Thr, Pro	Pro
Thr	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Ala	Gly, Thr, Pro, Ala, Ser	Gly, Ala
Val	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
Gly	Ala, Thr, Pro, Ser, Gly	Gly, Ala
Ile	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Phe	Trp, Phe,Tyr	Tyr, Phe
Tyr	Trp, Phe,Tyr	Phe, Tyr
Cys	Ser, Thr, Cys	Cys
His	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Gln	Glu, Asn, Asp, Gln	Asn, Gln
Asn	Glu, Asn, Asp, Gln	Asn, Gln
Lys	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Asp	Glu, Asn, Asp, Gln	Asp, Glu
Glu	Glu, Asn, Asp, Gln	Asp, Glu
Met	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Trp	Trp, Phe,Tyr	Trp

TABLE II

Amino Acid	Synonymous Group
Ser	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Arg	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-.Met, D-Ile, Orn, D-Om
Leu	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Pro	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Thr	D-Thr, Ser, D-Ser, allo-Thr, Met,D-Met, Met(O), D-Met(O), Val, D-Val
Ala	D-Ala, Gly, Aib, B-Ala, Acp, L-Cys, D-Cys
Val	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG
Gly	Ala, D-Ala, Pro, D-Pro, Aib, .beta.-Ala, Acp
Ile	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Phe	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Tyr	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Cys	D-Cys, S–Me–Cys, Met, D-Met, Thr, D-Thr
Gln	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Asn	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Lys	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Om
Asp	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Glu	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Met	D-Met, S–Me–Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

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CLAIMS

1. CXCL8 antagonists consisting of mutants of human mature CXCL8 (SEQ ID NO: 1), wherein at least the two basic residues Lysine 64 and Lysine 67 are substituted to Alanine, Glycine, Serine, Threonine, Proline, Glutammic Acic, Glutamine, Aspartic Acid, or Asparagine.
2. The antagonists of claim 1 wherein a third basic residue is substituted to Alanine, Glycine, Serine, Threonine, Proline, Glutammic Acic, Glutamine, Aspartic Acid, or Asparagine.
3. The antagonists of claim 2 having the sequence of CXCL8-1B3 (SEQ ID NO: 2), or of CXCL8-2B3 (SEQ ID NO: 3).
4. CXCL8 antagonists selected from:
   a) active mutants of the antagonists of claims from 1 to 3, in which one or more amino acids have been added, deleted, or substituted;
   b) peptide mimetics designed on the sequence and/or the structure of polypeptides or peptides of (a);
   c) polypeptides or peptides comprising the amino acid sequence of (a) or (b), and an amino acid sequence belonging to a protein sequence other than human mature CXCL8;
   d) active fractions, precursors, salts, derivatives, conjugates or complexes of (a), (b), or (c).
5. The antagonists of claim 4 wherein the one or more amino acids that have been added, deleted, or substituted in the mutants of (a) belong to the first six amino acids in the amino-terminal domain of the mature human CXCL8.

6. The antagonists of claim 4, wherein the mutants of (c) comprises the amino acid sequence belonging to one or more of these protein sequences: extracellular domains of membrane-bound protein, immunoglobulin constant region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins.

7. The antagonists of claim 4, wherein conjugates or complexes of (d) are formed with with a molecule chosen amongst radioactive labels, biotin, fluorescent labels, cytotoxic agents, or drug delivery agents.

8. DNA molecules comprising the DNA sequences coding for the antagonists of claims from 1 to 6, including nucleotide sequences substantially the same.

9. Expression vectors comprising the DNA molecules of claim 8.

10. A host cell transformed with a vector of claim 9.

11. Purified preparations that contain at least 1% of the CXCL8 antagonists of claims from 1 to 7, or of the DNA of claim 8 or 9.

12. Use of a CXCL8 antagonists of claims from 1 to 7, of the DNA of claim 8 or 9, or of the cells of claim 10, as active ingredients in pharmaceutical compositions for the treatment or prevention of CXCL8-related diseases.

13. Process of preparation of antagonists of claims from 1 to 7, comprising culturing the transformed cells of claim 10 and collecting the expressed proteins.

14. Pharmaceutical composition containing a CXCL8 antagonist of claims from 1 to 7, [illegible] of claim 9 or the cells of claim 10 as active ingredient.

antagonist of claims from 1 to 7, the DNA of claim 8 or 9, or the cells of claim 10, together with a pharmaceutically acceptable carrier.

16. Method for the treatment or prevention of a CXCL8-disease, comprising the administration of an effective amount of a CXCL8 antagonist of claims from 1 to 7, the DNA of claim 8 or 9, or the cells of claim 10.

ABSTRACT

Novel antagonists of the chemokine CXCL8 (also known as Interleukin-8) can be obtained by generating mutants having specific combinations of non-conservative substitutions of basic amino acids located in the C-terminal region. Compounds prepared in accordance with the present invention can be used to block CXCL8 activity *in vivo*, thereby providing therapeutic compositions for use in the treatment or prevention of CXCL8-related diseases.

Figure 1

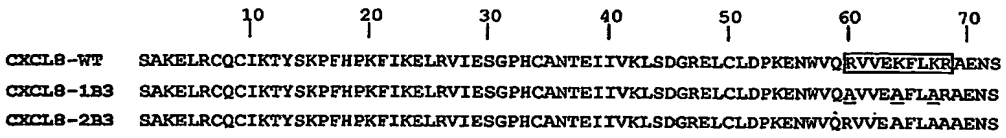
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Figure 2

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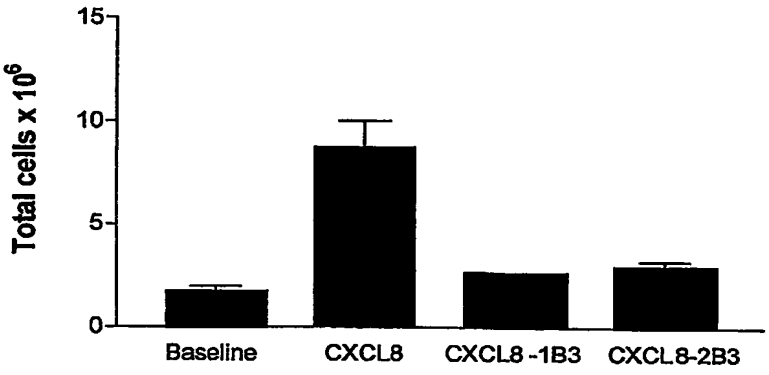
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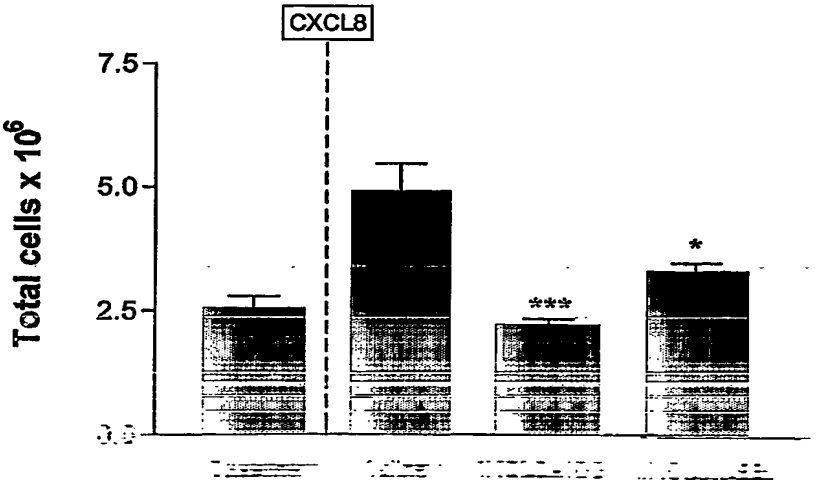
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